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Vaccine efficacy of a cell lysate with recombinant baculovirus-expressed feline infectious peritonitis (FIP) virus nucleocapsid protein against progression of FIP

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Abstract

The Type II feline infectious peritonitis virus (FIPV) infection of feline macrophages is enhanced by a monoclonal antibody (MAb) to the S protein of FIPV. This antibody-dependent enhancement (ADE) activity increased with the MAb that showed a neutralizing activity with feline kidney cells, suggesting that there was a distinct correlation between ADE activity and the neutralizing activity. The close association between enhancing and neutralizing epitopes is an obstacle to developing a vaccine containing only neutralizing epitopes without enhancing epitopes. In this study, we immunized cats with cell lysate with recombinant baculovirus-expressed N protein of the Type I FIPV strain KU-2 with an adjuvant and investigated its preventive effect on the progression of FIP. Cats immunized with this vaccine produced antibodies against FIPV virion-derived N protein but did not produce virus-neutralizing antibodies. A delayed type hypersensitivity skin response to N protein was observed in these vaccinated cats, showing that cell mediated immunity against the FIPV antigen was induced. When these vaccinated cats were challenged with a high dose of heterologous FIPV, the survival rate was 75% (6/8), while the survival rate in the control group immunized with SF-9 cell-derived antigen was 12.5% (1/8). This study showed that immunization with the cell lysate with baculovirus-expressed N protein was effective in preventing the progression of FIP without inducing ADE of FIPV infection in cats.

Keywords: Feline infectious peritonitis; Baculovirus; Vaccine; Nucleocapsid protein

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1. Introduction

Feline infectious peritonitis (FIP) is a virus-induced chronically progressive, immunologically-mediated and usually fatal disease in domestic and wild *Felidae*. The causative agent of this disease is FIP virus (FIPV) which belongs to the family *Coronaviridae*. To prevent FIP, various vaccines such as virulence-attenuated live or inactivated FIPV vaccine have been investigated, but no vaccine has exhibited a sufficient effect and the vaccines rather enhanced the onset of FIP (Woods and Pedersen, 1979; Pedersen et al., 1981; Pedersen and Black, 1983; Pedersen et al., 1984; Barlough et al., 1984; Pedersen, 1987; Stoddart et al., 1988). Intraperitoneal inoculation of virulent FIPV induced more severe clinical signs in anti-FIPV antibody-positive kittens and kittens that received passive immunization with serum or purified IgG from antibody-positive cats than in antibody-negative kittens (Pedersen and Boyle, 1980; Weiss and Scott, 1981). The antibody-induced enhancement of FIPV infection is a serious obstacle to the prevention of FIP by vaccination.

Generally, macrophages play an important role in the non-specific defense against viral infections. However, it is also known that some viruses bound to antibodies invade macrophages via the Fc region of the antibody and the Fc gamma receptor (FcγR) of the macrophage, and eventually, the antibody leads to the enhancement of infection. This phenomenon is called antibody-dependent enhancement (ADE) of virus infection (Halstead and O'Rourke, 1977). Macrophages are known to be one of the target cells for FIPV and it has been reported that the ADE of FIPV infection is induced by the same mechanism (Hohdatsu et al., 1991a; Corapi et al., 1992; Olsen et al., 1992; Hohdatsu et al., 1998).

FIPV consists of three major proteins, nucleocapsid (N) protein, transmembrane (M) protein and peplomer (S) protein. Among these proteins, neutralizing and ADE epitopes are mainly present on the S protein (Hohdatsu et al., 1991a; Corapi et al., 1992; Olsen et al., 1992). We previously reported that in vitro FIPV infection of feline alveolar macrophages is enhanced by a monoclonal antibody (MAb) to the S protein of FIPV. This ADE activity increased with the MAb that showed a neutralizing activity with feline kidney cells, suggesting that there was a distinct correlation between ADE activity and the neutralizing activity (Corapi et al., 1992; Olsen et al., 1992; Hohdatsu et al., 1993). The close association between enhancing and neutralizing epitopes is an obstacle to developing a vaccine containing only neutralizing epitopes without enhancing epitopes. Although a recombinant vaccine has been prepared by inserting the gene encoding S protein into vaccinia virus, the vaccine did not prevent FIPV infection and it rather enhanced the onset of FIP (Vennema et al., 1990).

The effects of recombinant vaccines prepared by inserting the genes encoding FIPV M or N proteins into vaccinia virus (Vennema et al., 1991) or raccoon poxvirus (Wasmoen et al., 1995) and a DNA vaccine containing the *IL-12* gene with the *M* and *N* genes (Glansbeek et al., 2002) on FIPV infection have been investigated. Among these studies, only the vaccine using recombinant raccoon poxvirus expressing the *N* gene (rRCNV–FIPVN) reported by Wasmoen et al. (1995) was effective against a low-dose FIPV challenge. However, since they challenged feline enteric coronavirus (FECV) orally after immunization with rRCNV–FIPVN, their study did not show the effect of the vaccine alone. Moreover, this vaccine was a recombinant live vaccine and its field application requires the resolution of many problems including its safety.

A temperature-sensitive mutant strain of FIPV has been produced, and appears to be both safe and efficacious against a low dose homologous experimental challenge (Christianson et al., 1989; Gerber et al., 1990). However, there is still controversy over the safety and efficacy of this vaccine in that protection may depend on the strain and dose of the challenge virus (Scott et al., 1992; Scott et al., 1995; McArdle et al., 1995). The vaccinated cats showed ADE when challenged with a high dose of a heterologous virus strain (Scott et al., 1992; Scott et al., 1995). Therefore, no vaccine is sufficiently safe and effective against FIPV infection at present.

In this study, we immunized cats with the cell lysate with baculovirus-expressed N protein of the Type I FIPV strain KU-2 with an adjuvant and investigated its preventive effect on the onset of FIP. The vaccinated cats produced antibodies against N protein but did not produce virus-neutralizing antibodies. Cell mediated immunity is considered to play an important role in the prevention of the onset of FIP. A delayed type hypersensitivity (DTH) skin response to N protein was observed in these vaccinated cats. When the vaccinated cats were challenged with a high dose of heterologous FIPV, the onset of FIP was prevented without the induction of ADE and the survival rate was 75%.

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda (Sf)-9 cells were grown in monolayers at 27 °C in TC-100 medium containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Felis catus whole fetus (fcwf)-4 cells were cultured as monolayers at 37 °C in a humidified atmosphere of 5% CO_2 in Eagle's minimum essential medium containing 50% Leibovitz's L-15 medium, 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. Type I FIPV strain KU-2 and Type II FIPV strain 79-1146 were grown in fcwf-4 cells at 37 °C. The FIPV KU-2 strain was isolated in our laboratory, and the FIPV 79-1146 strain was supplied by Dr. M.C. Horzinek of State University Utrecht, The Netherlands. A recombinant baculovirus expressing FIPV N protein was grown in SF-9 cells at 27 °C.

2.2. Cats

Anti-feline coronavirus (FCoV) antibody-negative specific pathogen-free cats aged 6–9 months were used. The cats were maintained in a temperature-controlled isolated facility.

2.3. Preparation of recombinant baculovirus-expressing the FIPV N protein

Genomic RNA was extracted from FIPV strain KU-2 infected culture fluid. The reverse transcription (RT) of genomic RNA and amplification of cDNA by polymerase chain reaction (PCR) were carried out using a standard technique with a DNA thermal cycler. RT-PCR primers were designed to cover the entire 1134 bp FIPV strain *KU-2N* gene (Accession no. AB086881). The PCR products were cloned into pFastBac1 plasmid. *Escherichia coli* DH10Bac strain was transfected with the recombinant plasmid DNA and cultured. The

white colonies grown were selected and the recombinant bacmid DNA was recovered. SF-9 cells were transfected with the recombinant bacmid DNA using cell fectin, and the culture supernatant was used in the experiment as recombinant baculovirus-expressed FIPV N protein.

2.4. Preparation of vaccine

SF-9 cells cultured for 2 days were inoculated with the recombinant baculovirus. After absorption for 1 h, serum-free TC-100 medium was added to the cells and the cells were cultured at 27 °C. After culture for 96 h, the infected cells were recovered and washed with PBS. One milliliter of RSB buffer containing 0.2% NP-40 (0.01 M NaCl, 0.0015 M $MgCl_2$, 0.01 M Tris–HCl, pH 7.4) was added to 1×10^7 cells and the cell suspension was kept at 4 °C for 30 min with occasional shaking. The cells were centrifuged at $800 \times g$ for 10 min. The precipitate was resuspended in PBS and used as recombinant N protein. Feline inactivated trivalent vaccine (Felidovac PCR; Intervet, The Netherlands), which is commercially available in Japan, was added to the recombinant N protein as an adjuvant. This feline inactivated trivalent vaccine contains 2% aluminum hydroxide gels and L80 as an adjuvant. The specificity of recombinant N protein and the amount of antigen were measured by enzyme-linked immunosorbent assay (ELISA) and Western immunoblotting using an anti-FIPV monoclonal antibody. ELISA and Western immunoblotting were performed by the method of Hohdatsu et al. (1991b).

SF-9 cell-derived antigen was prepared as the control antigen by NP-40 treatment of SF-9 cells on Day 4 of culture as in the recovery of recombinant N protein.

2.5. Purification of N protein from detergent-disrupted FIPV virions

The detergent-disrupted virions of FIPV strain 79-1146 purified by discontinuous sucrose density gradient ultracentrifugation were separated by SDS–PAGE. The position corresponding to the molecular weight of N protein was estimated from the positions of marker proteins, and the gel at the position was recovered. N protein was electrophoretically eluted from the recovered gel using protein elution apparatus (Maxyield GP; Atto Corp., Japan). The specificity of N protein was investigated using MAbs recognizing FIPV S, M, and N proteins (Hohdatsu et al., 1991b). The electrophoretically purified N protein was used as an antigen to detect the antibodies in ELISA and the measurement of the DTH skin response.

2.6. Design of immunization/challenge procedure

To evaluate the efficacy of the recombinant N protein vaccine, the same vaccination/challenge experiment was repeated twice. Eight SPF cats aged 6 months and eight SPF cats aged 7–9 months were used in the first and second experiments, respectively. In both experiments, four cats were subcutaneously vaccinated three times with 3-week intervals. As a challenge control, four cats received a subcutaneous administration of the SF-9 cell-derived antigen, which was prepared as the recombinant N protein described above, with the adjuvant. Four weeks after the third vaccination, all cats were challenged oronasally with 10^5 $TCID_{50}$ FIPV strain 79-1146. These cats were observed daily for clinical disease signs,

and the body temperature and body weight were measured every 3 days. In the second experiment, oropharyngeal swabs were obtained every 3 days for virus isolation. Serum was collected every 6 days for the detection of anti-FIPV antibodies.

2.7. Serological assays

Antibody responses to FIPV nucleocapsid were detected by ELISA using N protein electrophoretically purified from detergent-disrupted FIPV virions. FIPV-neutralizing activity in heat-inactivated cat sera were determined as described previously using fcwf-4 cells in a 96-well microplate assay (Hohdatsu et al., 1992). The antibody titer was expressed as the reciprocal of the highest dilution of serum that completely inhibited a viral cytopathic effect.

2.8. DTH skin response

The cell-mediated immune response to the FIPV antigen was measured by intradermal skin testing using N protein electrophoretically purified from detergent-disrupted FIPV virions. The left lateral abdomen of the vaccinated cats was shaved and disinfected with 70% ethanol, and 0.1 ml of 100 μg/ml antigen or PBS was intradermally injected into the each skin site. The diameter of swelling at the injection site was measured 24, 48, 72, and 96 h after injection using a caliper.

3. Results

The specificity of the recombinant baculovirus-expressed FIPV N protein was investigated by ELISA and Western immunoblotting using MAbs. In the ELISA, two-fold serial dilutions of the recombinant N protein were immobilized on 96-well flat bottom Microelisa plates and reacted with MAb F80-1 against S protein, MAb F18-2 against M protein, or MAb E22-2 against N protein. As shown in Fig. 1A, the recombinant N protein did not react with F80-1 or F18-2, and reacted only with E22-2, which recognizes N protein. Similarly, the recombinant N protein reacted only with E22-2 on Western immunoblotting and a specific band was detected at the position corresponding to 45 kDa. The amount of the recombinant N protein antigen per dose of the vaccine was measured by Western immunoblotting using MAb E22-2. The amount of the antigen detected as a positive band up to 16-fold dilution (16 U per dose) by Western immunoblotting was contained in one dose of the vaccine (Fig. 1B).

Humoral and cell-mediated immune responses to the FIPV antigens were investigated in the cats immunized with the recombinant N protein vaccine and the control cats. An increase in the ELISA value against FIPV virion-derived N protein was observed in Week 4 after the third immunization in all vaccinated cats in the first and second experiments (Fig. 2). However, neutralizing antibody titers against the homologous strain KU-2 and the challenge virus strain 79-1146 did not increase even after the third immunization, and the titer was 1:10 or lower in all vaccinated cats. In the control cats immunized with SF-9 cell-derived antigen, neither the ELISA value nor neutralizing antibody titer increased. Three weeks

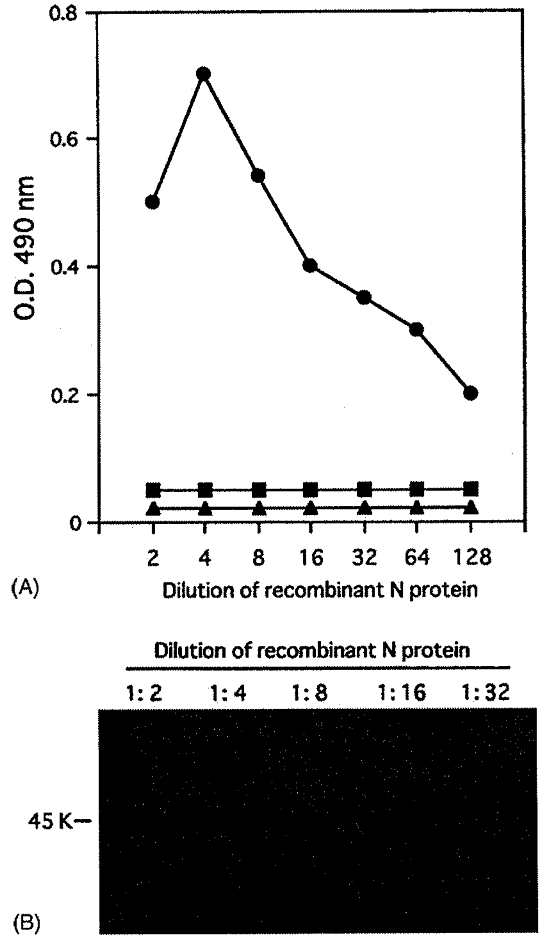

Fig. 1. Specificity of recombinant baculovirus-expressed FIPV N protein. (A) The specificity of recombinant N protein was investigated by ELISA using anti-FIPV MAbs. (●) Reactivity of MAb E22-2 recognizing FIPV N protein. (■) Reactivity of MAb F18-2 recognizing FIPV M protein. (▲) Reactivity of MAb F80-1 recognizing FIPV S protein. (B) Visualization of recombinant N protein on a Western blot after staining with MAb E22-2 recognizing FIPV N protein.

after the third immunization, 0.1 ml of FIPV virion-derived N protein was intracutaneously injected into the vaccinated cats, and the induction of DTH skin response to the FIPV antigen was investigated. In the four vaccinated animals in the first experiment, swelling at the N protein-injected skin site was observed after 24 h and the swelling persisted until 72 h. In these cats, no swelling was observed at the PBS-injected skin site. In the second experiment, swelling was also observed at the N protein-injected skin site in the four vaccinated cats. In cat no. O-3 in the second experiment, transient swelling was observed at the PBS-injected skin site after 72 h. Fig. 3 shows the diameters of the swelling measured using a caliper in the cats in the first and second experiments.

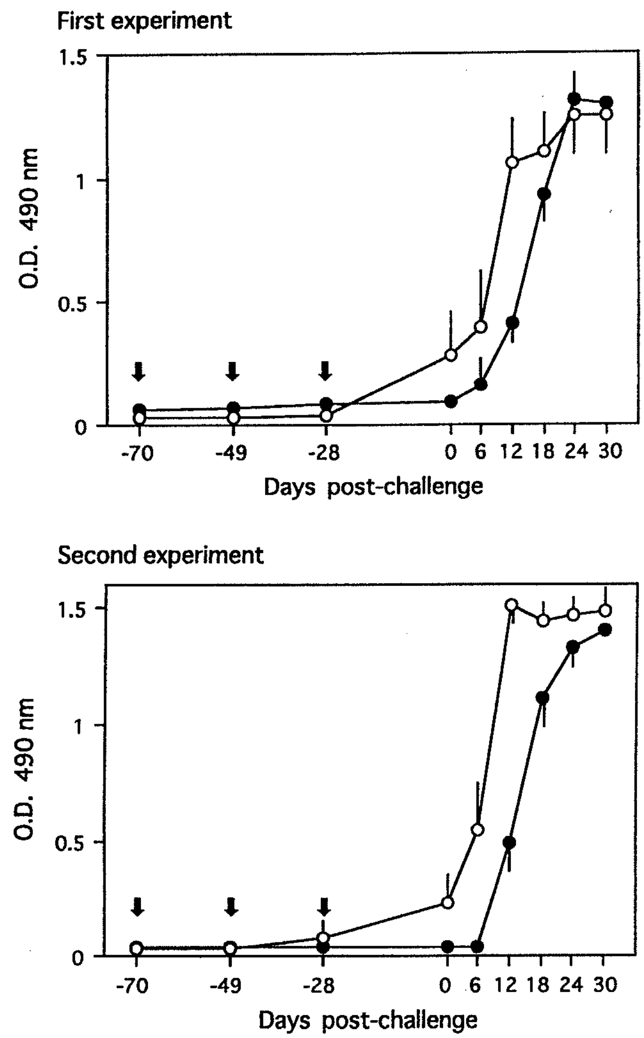

Fig. 2. Antibody levels in vaccinated and control cats before and after FIPV challenge. Sera collected at the indicated times were tested by ELISA using N protein electrophoretically purified from detergent-disrupted FIPV virions. Arrows indicate vaccine doses. (○) Mean ± S.D. of ELISA OD-values in vaccinated cats. (●) Mean ± S.D. of ELISA OD-values in control cats.

Four weeks after the third vaccination, all cats were challenged oronasally with 10^5 $TCID_{50}$ FIPV strain 79-1146. The antibody response to N protein, the neutralizing antibody titer against the challenge virus, changes in body weight, and the survival curves are shown in Table 1, Fig. 2, Figs. 4 and 5, respectively. In all vaccinated cats, the ELISA value against N protein began to increase on Day 6 after the challenge and the antibody responded earlier than that in the control cats (Fig. 2). The anti-challenge virus (strain 79-1146) neutralizing antibody production converted to positive on Day 12 after the challenge in the vaccination

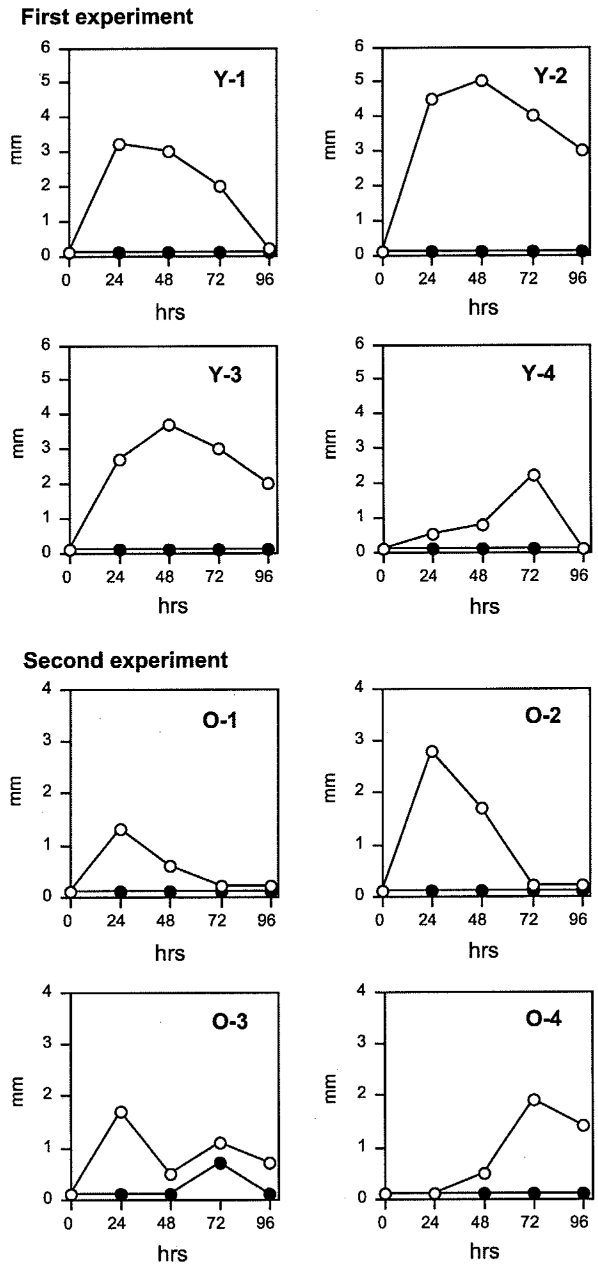

Fig. 3. Delayed-type hypersensitivity (DTH) skin response to FIPV N protein in vaccinated cats. Skin tests were performed by intra-dermally injecting either 0.1 ml of N protein electrophoretically purified from detergent-disrupted FIPV virions or 0.1 ml of PBS. The diameter of swelling at the injection site was measured 24, 48, 72, and 96 h after injection using a caliper. (○) N protein-injected skin site and (●) PBS-injected skin site.

Table 1
Neutralization antibody titer and onset of death following FIPV challenge

Group	Vaccine	Cat no.	Days post-challenge											Day of death
			0	12	18	19	23	26	29	31	44	48	60	
First exp.	Recombinant N protein	Y-1	<10	20	80								6400	Survival
		Y-2	<10	160	320								6400	Survival
		Y-3	<10	40	160							6400		48
		Y-4	<10	40	80								1600	Survival
	Controls (SF-9 cells)	Y-5	<10	80	320						6400			44
		Y-6	<10	80	160					1600				31
		Y-7	<10	80	160		800							23
		Y-8	<10	80	160				1600					26
Second exp.	Recombinant N protein	O-1	<10	40		200								19
		O-2	<10	20	80								6400	Survival
		O-3	<10	20	80								3200	Survival
		O-4	<10	10	40								3200	Survival
	Controls (SF-9 cells)	O-5	<10	10	40			400						29
		O-6	<10	40	160								3200	60
		O-7	<10	40	160								6400	Survival
		O-8	<10	40	80		200							23

and control groups, showing no significant difference between the two groups (Table 1). Samples obtained from oropharyngeal swabs collected every 3 days were inoculated with fcwf-4 cells to isolate the virus. The virus was isolated 3–9 days after the challenge from all cats in the vaccination and control groups, showing no significant difference in virus isolation. In the first experiment, the body weight gradually decreased and severe clinical disease developed in all cats in the control group, and the animals were euthanized 23–44 days after the FIPV challenge. The post-mortem examination findings were consistent with systemic pathological FIP disease. In contrast, in the vaccination group, cat Y-3 developed FIP but the other three animals developed no clinical signs of FIP during the 90-day observation period (Figs. 4 and 5). Similarly, in the second experiment, three of four animals in the control group developed systemic pathological FIP disease 23–60 days after the FIPV challenge and were euthanized. In the vaccination group, only cat O-1 developed FIP but the other three animals tolerated the challenge (Figs. 4 and 5). Combining the results of the first and second experiments, the survival rates were 75% (6/8) and 12.5% (1/8) for the immunized and control groups, respectively. These survival rates were analyzed using the χ^2-test, and there was a significant difference ($P < 0.05$).

4. Discussion

This study confirmed that immunization of cats with baculovirus-expressed N protein was effective in preventing the progression of FIP following a high-dose (10^5 $TCID_{50}$) FIPV

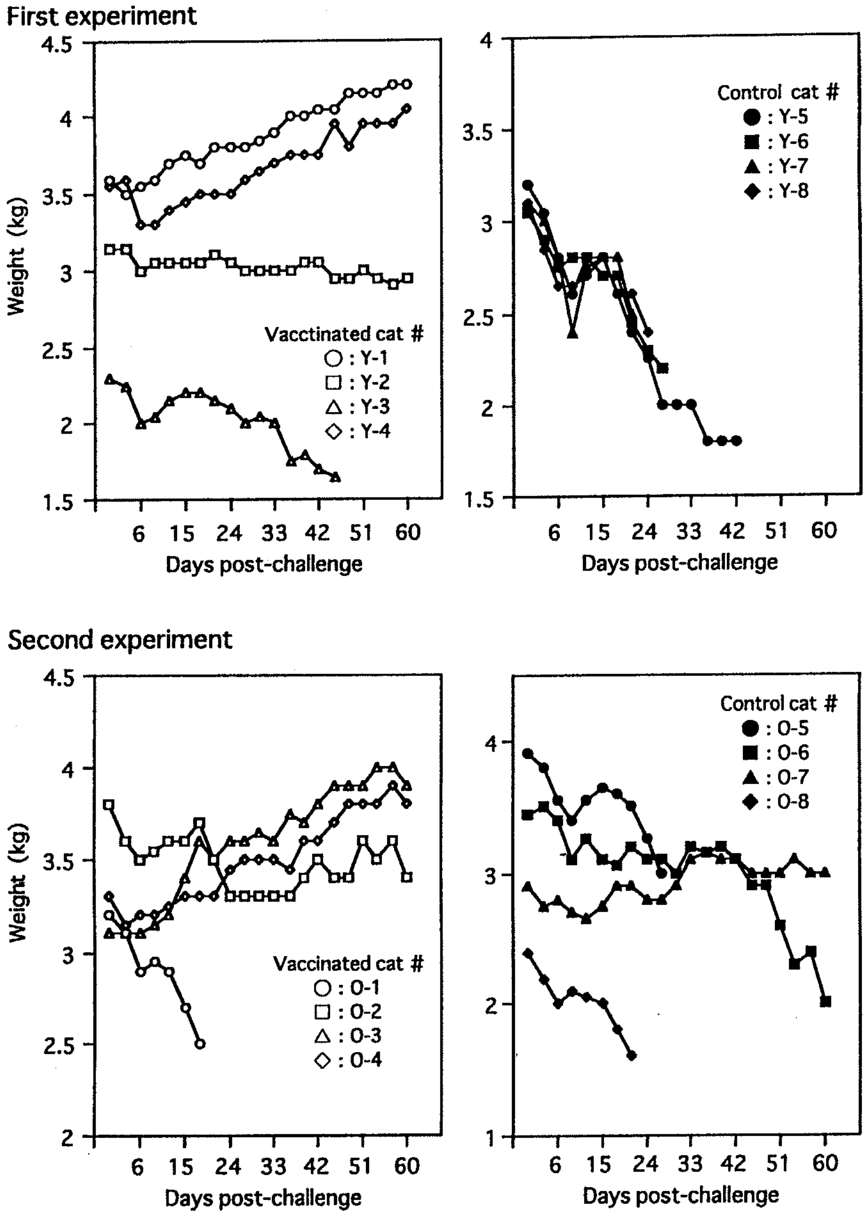

Fig. 4. Weight curves following FIPV challenge.

challenge. In cats immunized with this vaccine, the inhibition of FIP progression attained a survival rate of 75%. As described in the Introduction, Wasmoen et al. (1995) reported that in cats infected with FECV after immunization with recombinant racoon poxvuris expressing the *N* gene, the onset of FIP was 100% inhibited against a low-dose virulent

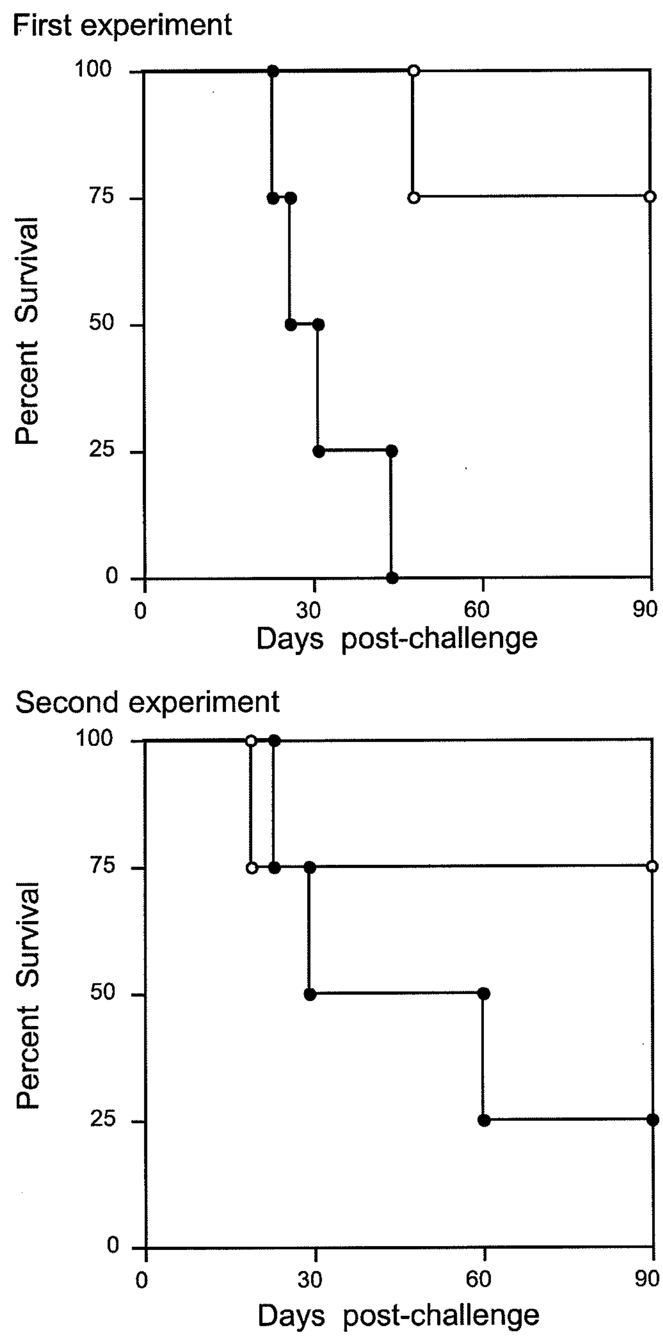

Fig. 5. Survival curves following FIPV challenge. (○) Vaccinates and (●) controls.

FIPV challenge (10^3 $TCID_{50}$). However, their study did not show the effect of the vaccine alone. Our study is the first to report that recombinant N protein alone attained a survival rate of 75% against a high-dose virulent FIPV challenge. Although we used Type I FIPV strain KU-2-derived recombinant N protein as the antigen, the vaccine also inhibited the onset of FIP following a challenge with Type II FIPV, 79-1146 strain. It is still unclear whether this immunization is effective against the same serotype FIPV challenge. Since the pathogenicity of oronasal challenge of KU-2 strain, which may be the natural route of infection, is weak, the vaccine effect on KU-2 strain could not be evaluated using the onset of FIP as the index in this study. Since Type I FIPV/FECV infection is dominant in the field (Hohdatsu et al., 1992), it is necessary to investigate the protective effects against Type I FIPV infection in the future. However, the amino acid sequence of N protein is conserved relatively well among FIPV strains (90% or higher homology; Motokawa et al., 1996) and infection with any type of FIPV may be controlled by use of this protein. No enhancing epitope present on N protein has been reported and no ADE was observed in this study, confirming that this protein is safe. In contrast, Gene analysis of S protein has shown that the amino acid sequences of Types I and II FIPV are very different (about 46% homology; Motokawa et al., 1996), suggesting that it is difficult to achieve an effect on infections with the two types of FIPV when S protein is selected for the vaccine antigen.

There was no difference in the virus isolation from oropharyngeal swabs collected after virulent FIPV challenge between the vaccinated and control cats in the second experiment, showing that FIPV infection was not prevented. However, virulent FIPV challenge-induced progression to FIP was prevented in the vaccinated cats. Cellular immunity is considered important for the prevention of progression to FIP (Pedersen, 1987). The vaccinated cats developed a DTH-skin response to N protein, suggesting that cell mediated immunity against the FIPV antigen was induced. Weiss and Cox (1988, 1989) reported that a strong DTH skin response to FIPV antigen was induced in cats that survived FIPV infection after FIPV challenge-exposure. Although the data are not shown, we observed no DTH response to virion-derived N protein in anti-FCoV antibody-negative SPF cats or cats that developed FIP, while cats that tolerated the FIPV challenge-exposure and FECV-infected cats exhibited a positive reaction. These findings strongly suggest that a DTH response to FIPV is associated with increased resistance to disease.

Generally, an inactivated vaccine induces humoral immunity because the vaccine is recognized as an exogenous protein, but the induction of cellular immunity is difficult. However, it has been found that antigens incorporated into immunostimulating complexes or encapsulated into certain liposomes can stimulate cytotoxic T lymphocyte (CTL) immunity (Takahashi et al., 1990; Reddy et al., 1992). In addition, Kovacsovics-Bankowski et al. (1993) reported that soluble antigen conjugated to beads more efficiently induced CTL immunity than immunization with soluble antigen alone. It is considered that conversion of soluble to insoluble antigen by binding to beads increases phagocytosis of the antigen by antigen-presenting cells (APC), resulting in activation of MHC class I presenting pathway of exogenous proteins. The recombinant baculovirus-expressed FIPV N protein used in this study was insoluble antigen prepared as follows: recombinant baculovirus-infected SF9 cells were disrupted in RSB buffer containing 0.2% NP-40 and centrifuged at $800 \times g$ for 10 min, and the precipitate was resuspended in PBS. Use of insoluble N protein as the vaccine antigen may have induced cellular immunity including DTH response more efficiently. The

antigen prepared as described above may have contained baculovirus-derived antigens. It has recently been reported that baculovirus itself could induce non-specific anti-viral effects in mammals (Gronowski et al., 1999). Therefore, there is a possibility that baculovirus-derived antigen(s) has some synergetic effects on the successful protection.

The vaccine used in this study contained a high concentration of recombinant N protein (16 U on Western immunoblotting) and it prevented the onset of FIP without inducing ADE in a high-dose FIPV challenge. The vaccine may more effectively prevent the onset of FIP following a low-dose virus challenge close to spontaneous infection.

Acknowledgements

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